Journal of the Korean Magnetic Resonance Society **2018**, *22*, 91-100
DOI 10.6564/JKMRS.2018.22.4.091

Assessments in biocides with omics approaches to ecosystem

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Received Nov 16, 2017; Revised Nov 30, 2018; Accepted Dec 1, 2018

Abstract Benzisothiazolinone (BIT) is the preservative that is widely used in industrial and household products. In this study, zebrafish (*Danio rerio*) was exposed to BIT at different concentrations (control, 0.5 g/L, 1.0 g/L and 2.0 g/L) for 72 hours. The techniques of nuclear magnetic resonance (NMR) spectroscopy were applied to analyze the effects of BIT on zebrafish. The advantages of NMR are the minimal sample preparation and high reproducibility of experimental results. With the multivariate statistical analysis, dimethylamine, N-acetylaspartate, glycine and histidine were identified as an important metabolite in differentiating between the control and BIT-exposed group. This study will improve the understanding the metabolite changes in the zebrafish in response to BIT exposure.

Keywords High resolution-magic angle spinning, nuclear magnetic resonance spectroscopy, metabolomics, *Danio rerio*, benzisothiazolinone

Introduction

Biocidal substances are used to prevent the growth of a microorganism, and benzisothiazolinone (BIT) is one of the biocides that are most frequently used as preservatives1 in industrial and household products such as water-based toys,[2] hobby paints and finger paints.[3] There are no obvious restrictions on the use of BIT in household care products and other consumer products. Furthermore, safety for the substances is unclear.[4] So far, just few studies on the risk of BIT have been conducted. Therefore, it is necessary to evaluate the risk from the use of BIT.

In order to examine the risk of BIT, zebrafish (Danio rerio) was used for this study. Zebrafish is generally used for the toxicological study as it has short generation time with inexpensive maintenance.[5] Relatively high sensitivity of zebrafish to chemical exposure compared to other animal models is an advantage, and its similar genetic information to human is also a merit.[6-8] Thus, zebrafish is an adequate model for the current study.

We herein introduced nuclear magnetic resonance (NMR)-based metabolomics to investigate the response of zebrafish to the BIT. Metabolomics is helpful in the field of ecotoxicology[9] and also useful to study the effect of chemical exposure in zebrafish. Profiling of metabolic can be used to detect the physiological changes in zebrafish caused by BIT. NMR and mass spectrometry are the most widely used techniques for the analysis of the metabolite in zebrafish. NMR spectroscopy has several merits: it is the only approach which does not require a separation of the analytes, and the sample can be re-used for further study.[10] The main advantages of

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NMR are in the high reproducibility and simple sample preparation.[11,12] For these reasons, NMR turned out to be an appropriate tool to analyze the metabolite in fish. Thus, we aim to study the response and metabolic changes in zebrafish exposed to BIT using ^{1}H-NMR based metabolomics.

Experimental Methods

Exposure - Adult male zebrafish (*Danio rerio*) were purchased from Green Fish (Seoul, Korea). The body mass of fish was in the range of 0.8±0.3 g. The fish was acclimated for two weeks before the experiment. The fish were fed twice a day and changed twice a week during the acclimation period. The fish were maintained in 60-L dechlorinated water at 28±1 ℃ with 14/10-h light/dark cycle. Following the OECD guideline for acute toxicity test, the zebrafish were not fed for one day before the exposure.[13] Twenty zebrafish were randomly selected for the experiment. BIT was purchased from sigma Aldrich (Darmstadt, Germany). Five each male zebrafish was in a 5-L per tank and exposed to control, 0.5 g/L, 1.0 g/L and 2.0 g/L of BIT for 72 h. The photoperiod and the temperature were kept the same during the experiment. After the exposure, the fish were rinsed for an hour in order to remove any possible residual of BIT on the fish. Then, they were quickly frozen with liquid nitrogen and lyophilized overnight.

Sample preparation for NMR measurement - Completely dried zebrafish were obtained with the lyophilization. The dried fish was ground into a fine powder and well homogenized with mortar and pestle. Metabolites were extracted using Bligh and Dyer's method.[14] Methanol, distilled water and chloroform were used in the ratio of 1.6:1.4:1.6 to extract the aqueous metabolites. The extracts were then centrifuged at 3,000 rpm for 10 min at 4 ℃ to separate the aqueous layers. The process of lyophilization was performed overnight once again to remove any solvent in the aqueous layers. 700 μL of deuterium oxide (99% deuterium) which includes 2 mM 3-(Trimethylsily)propionic-2,2,3,3-d_4 acid sodium salt (TSP-d_4) was added to prepare metabolite samples which were completely dissolved in aqueous solutions. TSP-d_4 was added as a reference for the chemical shift (0.00 ppm). 5-mm NMR tubes were used for all NMR measurements.

NMR analysis - The ^{1}H-NMR spectra were acquired using a 600 MHz Agilent NMR spectrometer (Agilent Technologies, Palo Alto, CA, USA). A Carr-Pur-Cell-Meiboom-Gill (CPMG) pulse sequence was used to suppress the high molecular compounds signal.[15] The spectra were measured 9.8 μs of 90° pulse, 1.5 s of relaxation delay and 3 s of acquisition time. The total acquisition time was 13 mins, and 128 scans were acquired in each sample. The peak of TSP-d_4 at 0.00 ppm was used for the reference to calibrate the chemical shift.

For the multivariate statistical analysis, each ^{1}H -NMR spectrum was phased, and baseline was manually corrected. The size of the bin was divided into 0.001 ppm and the spectra were binned from 0.5 to 10.0 ppm. Then, binned data were normalized to the total area. The water peak region (4.68-4.90 ppm) and methanol peak region (3.32-3.36 ppm) were excluded. Binning was conducted using Chenomx NMR suite 7.1 (Chenomx Inc., Edmonton, AB, Canada). The result of binning data was imported to Matlab software, and alignment was sequentially performed using the icoshift algorithm of MATLAB R2013b (The Mathworks, Natick, MA, USA). Next, the aligned binning results were imported to SIMCA-P+ 12.0 (Umetrics, Umeå, Sweden). The principal component analysis, partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis were conducted using the SIMCA-P+ 12.0 software.

Assignment of ^{1}H-NMR spectra and quantification of metabolites were performed using the Chenomx NMR suite 7.1 software (Chenomx Inc., Edmonton, AB, Canada). Each peak of the ^{1}H-NMR spectrum was matched to the library database in Chenomx NMR suite 600 MHz, and compared to the peak of 2 mM of TSP-d_4 at 0 ppm.

Results

^{1}H-NMR spectroscopy was used to analyze the metabolites in aqueous extracts from zebrafish. Figure 1 shows the ^{1}H-NMR CPMG spectrum of zebrafish. The total of 55 metabolites was assigned and quantified: the amino acids (e.g., alanine, glycine, histidine, leucine, methionine, phenylalanine and valine), organic acids (e.g., acetate, lactate, pyruvate and succinate) and sugars (e.g., glucose). Figure 2 shows the box plots of the assigned 55 metabolites in zebrafish exposed to BIT. The diagram shows the distribution of input data values before and after normalization. The curved plot shows the concentration distributions of all compounds. The below density plots of 55 metabolites from BIT-treated zebrafish were compared to control (i.e., kernel density estimation). The measured concentrations were normalized using log-transformation followed by pareto-scaling.

Most studies in metabolomics have complex multivariate datasets with different correlations between the measured metabolite levels.[16] In order to find the metabolic biomarkers, multivariate discrimination model was introduced to compare the spectral patterns between the control and BIT exposed group. In metabolomics, PLS-DA is one of the methods that are mostly used in metabolomics.[17,18] In this study, PLS-DA was applied to improve the separation between the BIT treated group and the control (Fig. 3).

Metabolites with Variable Importance in Projection (VIP) scores of >1 for the BIT-treated group compared to the control were evaluated based on the PLS-DA value (Fig. 4). The VIP plot displays the most important metabolite features (top 15). Colored boxes on the right indicate the relative concentration of the corresponding metabolite for control and BIT exposed group. The red color represents an increase in the logarithmic transformation of concentration in metabolite, whereas the green represents a decrease. Each variable was scaled by the mean and the standard deviation value. As a result, dimethylamine (DMA), glycine and histidine were decreased compared to control. The VIP scores reveal the metabolites importance in the PLS model. A VIP score of 2.0 or a higher value indicates the significance. Thus, DMA, glycine and histidine were the significant metabolites.

For assessment of the performance in the diagnostic test, the specificity and the sensitivity are generally used.[19] The sensitivity is a way of measuring how well the model can separate the samples between the control and exposed group, whereas the specificity shows how accurately and precisely the model predicts the exposed group from the control.[16] The Receiver Operator Characteristic (ROC) combines the specificity and the sensitivity.[20,21] The ROC can be drawn by putting the sensitivity against 1-specificity. Then, the ROC curve provides a spectrum of performance assessments and the area under the ROC (AUROC) which is widely used as a diagnostic tool of PLS-DA models.[16] The value of 1 indicates the perfect separation between the exposed group and the control, and values below 0.5 indicate poor discrimination. In this study, DMA and NAA showed reliable results, and their ROC values were 0.96 and 0.92 respectively (Fig. 5). Two p-values were both less than 0.05. Using a PLS-DA model with AUROC as a diagnostic tool was powerful in discriminating the small differences between the control and the BIT-exposed group. Amounts of DMA decreased compared to the control while amounts of NAA increased. To sum up all the multivariate statistical analysis, DMA and NAA were the significant metabolites on the basis of ROC results. DMA, glycine and histidine were the major metabolite in the VIP plot.

Discussion

DMA is an organic osmolyte[22] and the decreased in DMA level in BIT-treated zebrafish are related to the changes in the environment of osmoregulation. In the study of Japanese rice fish (*Oryzias latipes*), the changes in the DMA level indicated that the osmoregulation have been affected.[23] In addition, the changes in the level of DMA content disturbed the lipid metabolism in the embryos of zebrafish.[24] Thus,

decreased content in DMA in adult zebrafish might have been associated with the lipid metabolism. NAA is known to be a brain metabolite and the changes in the level of NAA in zebrafish indicate that the brain metabolism might be affected.[25] NAA was found to be localized in neurons of the brain of a rat, therefore it was generally known as a neuronal marker.[26,27] The studies in the other animals show that mitochondrial energy metabolism might affect the changes in the NAA levels.[26,28] Levels of NAA reflect the neuronal metabolism which may be related to the neuronal dysfunction.[24]

Glycine, the neurotransmitter, plays an important role in the spinal cord network function in many vertebrate systems.[29] Glycine combines the left and the right activity in the spinal cord in order to generate the muscle contractions of coordinated motion.[30-33] Downes et al. suggests that glycine is necessary for spontaneous motion and touch-evoked tail coiling behavior in the early development of zebrafish larvae.[29] Histidine is known to be a precursor for neurohormones and neurotransmitters which is helpful for the operation of the histaminergic system.[34,35] Histidine and glycine are the significant metabolites to differentiate between the control and Alzheimer's disease patients.[36] Decreases in the content of glycine was observed in parkinsonian patients.[37] In this study, the content of glycine decreased, and, thus, the changes in the glycine content might indicate the neurodegenerative disease. Overall, the metabolites of BIT-treated zebrafish were related to the lipid and the neuronal metabolism. In addition, the use of zebrafish might be appropriate for the study on the effect of neurological diseases.

In this study, ^{1}H-NMR based metabolomics was applied to assess the potential risk of BIT in zebrafish. Metabolomics was introduced to observe the metabolic changes in zebrafish after the exposure to BIT for 72 hours. Results of the multivariate statistical analysis showed good separation between the control and the exposed group. DMA, NAA, histidine and glycine were the significant metabolites.

With ^{1}H-NMR based metabolomics, it was successful to detect the metabolic responses in BIT-treated zebrafish. The majority of metabolites which were detected was involved in the neurological disease. For further studies on the correlation with the neurological disease, the dissected brain in zebrafish is required to be analyzed

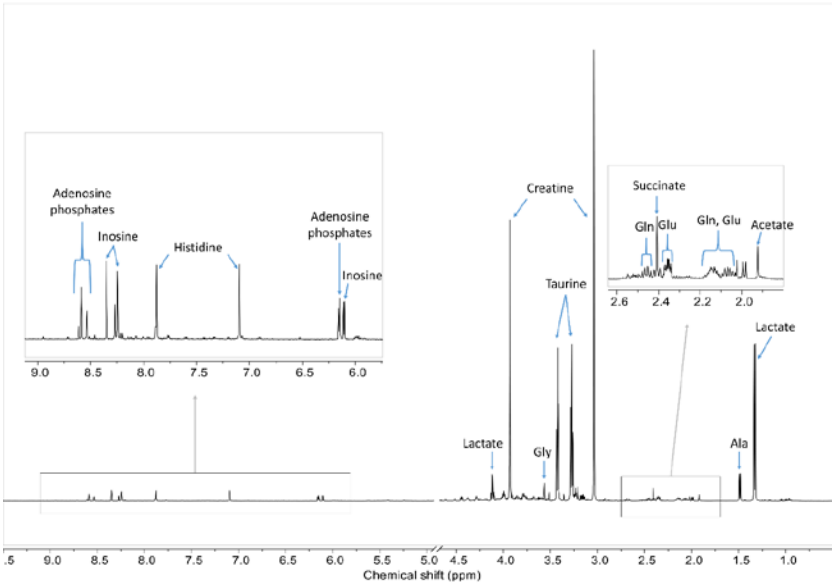

Figure 1. Representative ^{1}H-NMR spectrum of aqueous extracts of zebrafish. High and low field spectra were magnified. Ala, Gln and Glu indicate alanine, glutamine and glutamate, respectively.

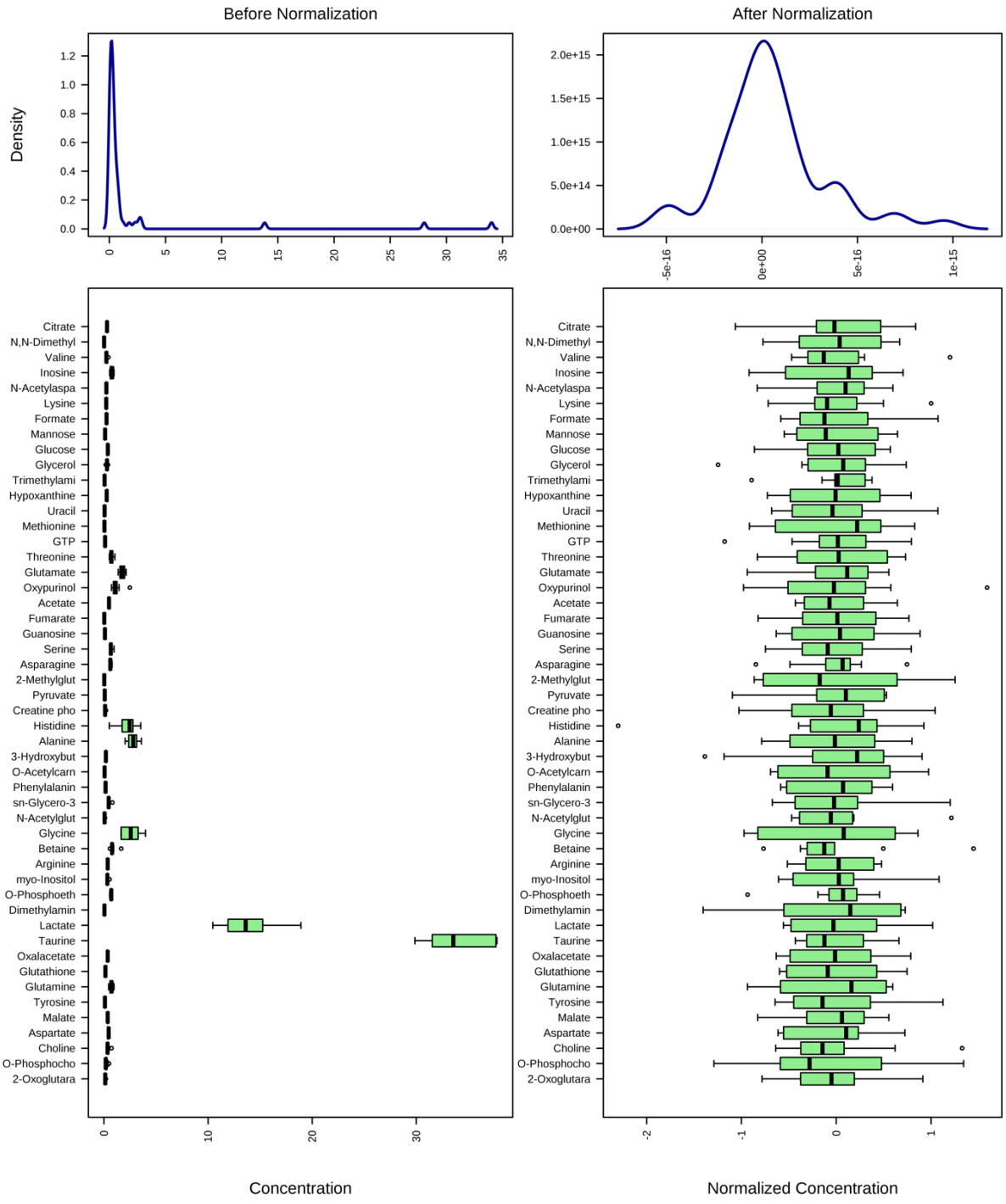

Figure 2. Data normalization plot (control-based z-score).

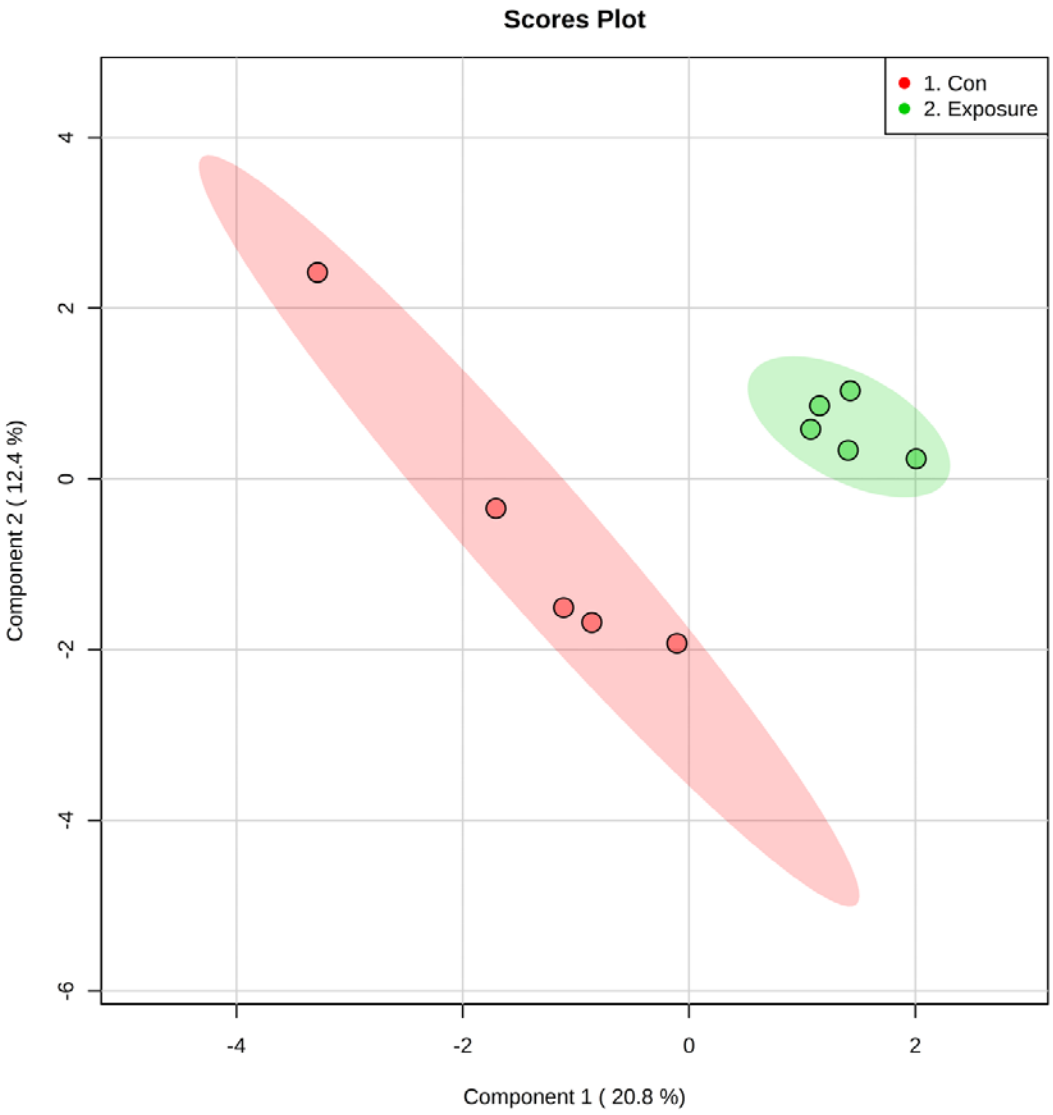

Figure 2. PLS-DA score plot of zebrafish exposed to BIT (●, control; ●, exposure).

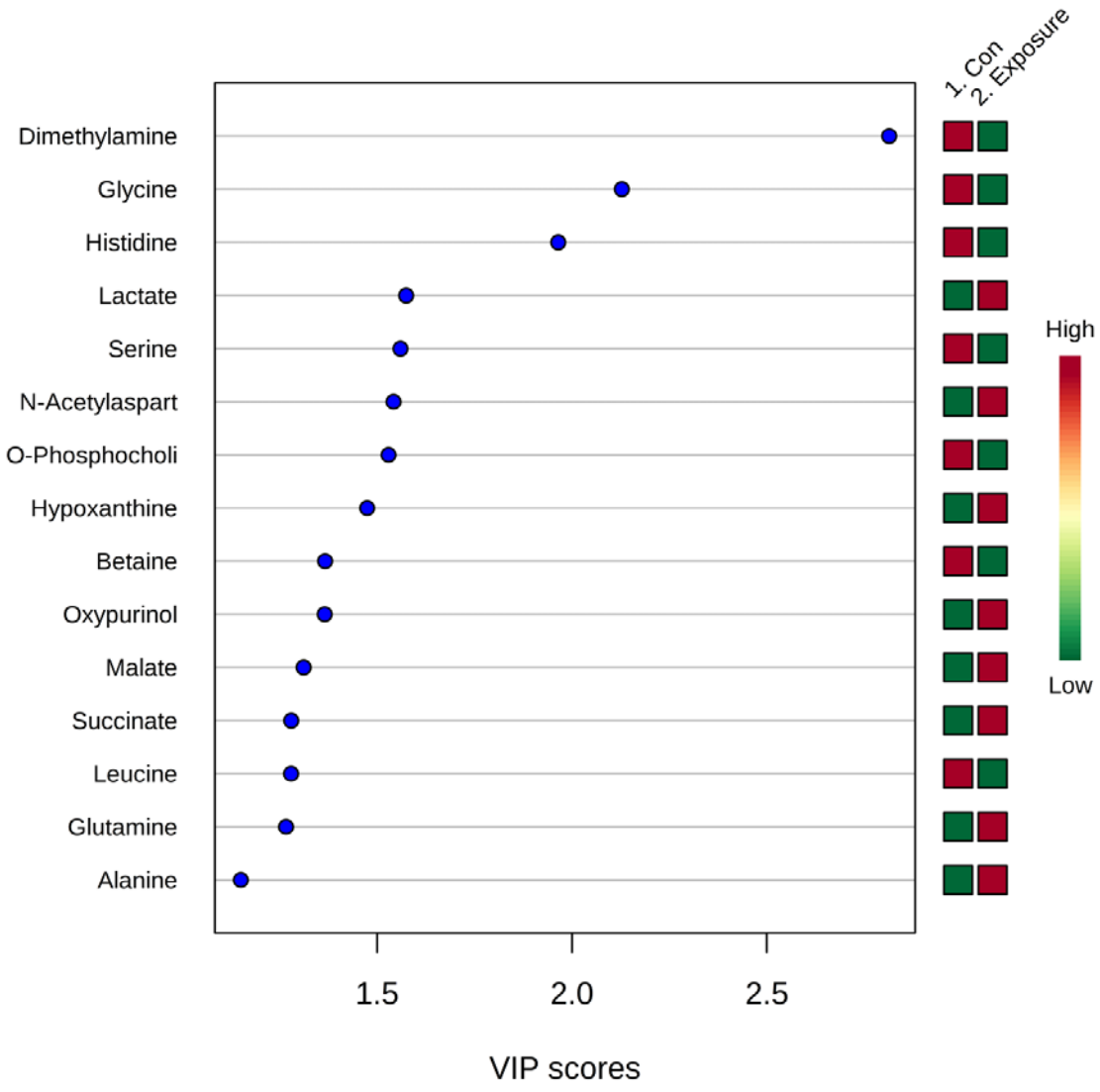

Figure 4. VIP plot. The VIP plot displays the top 15 most important metabolite features identified by PLS-DA. (Metabolites of VIP > 1).

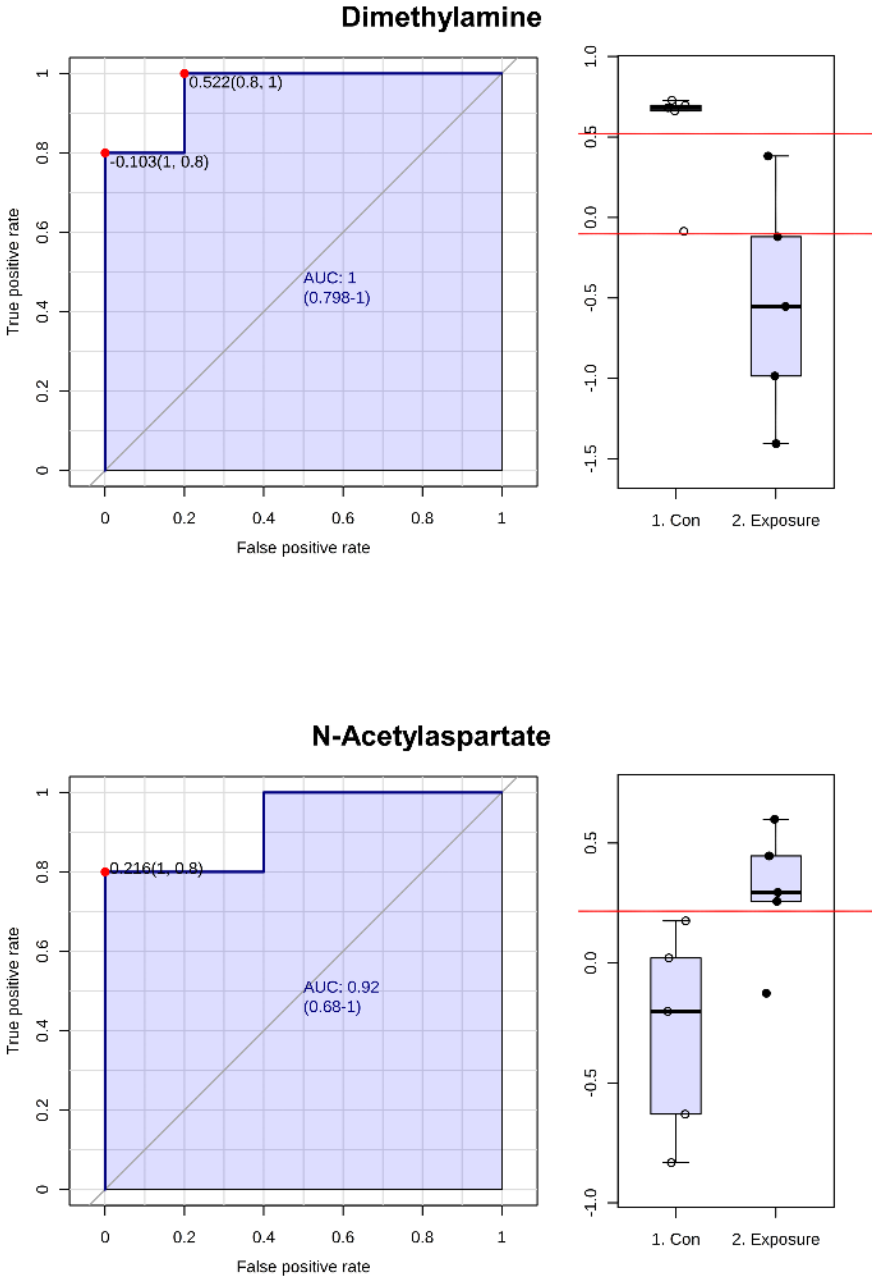

Figure 5. ROC curve. The ROC curves are the comparison of control and BIT exposed group. AUC values: Dimethylamine (0.96) and N-Acetylaspartate (0.92).

Acknowledgements

This work was supported by a 2-Year Research Grant of Pusan National University.

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